Effect of Water Treatment Residuals and Microcystin on Soil Chemical Properties, Soil Arylsulfatase Activity and Microbial Community Composition

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ABSTRACT

Microcystins (MCs) are monocyclic heptapeptides that are produced by the cyanobacteria, *Microcystis,* and have high structural variability. They are hepatotoxins and therefore, pose health risks to humans and animals who consume them. Application of MC contaminated water treatment residuals (WTR) to agricultural fields can potentially pose a threat to the soil ecosystem in terms of soil health and food production. Therefore, developing a method to quantify the total concentration of MC in WTR is needed in order to determine the fate and toxicity of MC in the environment. In this study, a MC extraction method was successfully tested, and two out of eight WTR samples analyzed (WTR 2 and 6) had the highest concentrations of MC while WTR 3 had no detectable MC. To determine the effect of MC on soil microbial properties, a 4-week incubation study, using a clay loam and sandy loam soil from northwestern Ohio, was conducted. The treatments were: WTR (no MC), WTR+MC, and MC. The treatments were analyzed for soil chemical properties, arylsulfatase activity, and microbial community composition by microbial phospholipid fatty acids (PLFAs). In both soils, the pH and EC significantly increased in all treated soils, whereas available P content significantly decreased in all treatments. Following the addition of MC in the sandy loam soil, arylsulfatase activity significantly increased. Application of MC and WTR+MC to both soils significantly increased total PLFA, gram-negative bacteria, and total fungal concentrations. The WTR treatment had a significant effect on gram-negative bacteria in the sandy loam. The gram-negative to gram-positive bacteria biomass ratio significantly increased in all the treatments in the clay loam and sandy loam. In addition, each treatment significantly decreased the microbial PLFA stress ratio (17:0cyc and c19:0cyc divided by 16:1w7c and 18:1w7c) in the clay loam and sandy loam. The results indicate that the application of MC, with or without WTR, stimulates the soil microbial community.

1. Introduction

With increasing anthropogenic impacts further stimulating climate change, harmful algal blooms are potentially increasing in size and toxicity in eutrophic waters. Dominated primarily by the cyanobacteria species *Microcystis*, but also *Planktothrix* and *Anabaena*, microcystin (MC), a hepatotoxin, is released into the aquatic environment. Microcystins are monocyclic heptapeptides with over 200 structural congeners (Cao et al., 2017; Cao et al., 2018). They have high structural variability due to 1) their ability to make side-chain modifications; 2) their capability to change amino acids in positions two and four of the ring; and 3) their variations in the Adda moiety (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca4,6-dienoic acid) (Corbel et al., 2015). They are considered to be recalcitrant substances because their cyclic structure is difficult for microorganisms to break down (Xiang et al., 2019). Of all the reported variants, MC with the amino acids leucine (L) and arginine (R) at positions 2 and 4 (MC-LR), respectively, is the most commonly found and most potent (Cao et al., 2017).

During cell lysis, *Microcystis* releases the harmful MCs into the aquatic ecosystem. From there, there are multiple ways in which MCs can enter the soil ecosystem. For example, the biomass from the blooms is often added to the soil as an organic fertilizer. Additionally, irrigation water, especially when sourced from areas that experience harmful algal blooms, contain MCs (Cao et al., 2017). Lastly, the use of water treatment residuals (WTR) in agricultural fields can introduce large quantities of microcystin into the environment as they become concentrated in the WTR byproduct during the water treatment process (Ai et al., 2020).

Water treatment residual is primarily composed of sediment, activated carbon, polymers, and aluminum oxide (Gallimore et al., 1999). Some of the advantages of WTR application to soil include an increase in porosity, water holding capacity, and nutrients. In addition, WTR has the

ability to affect soil pH, available phosphorus, and anionic concentration (Ai et al., 2020). Due to the presence of aluminum oxide and its amorphous nature, WTR is capable of binding large quantities of anions, including dissolved P, through adsorption and precipitation mechanisms. Therefore, application and incorporation of WTR into soil can reduce the amount of dissolved P, reducing the amount of P runoff into freshwater ecosystems, which is especially important when excess amounts of P have been applied to agricultural fields (Gallimore et al., 1999; Ippolito et al., 2011). As production of WTR accumulates, the use of it as an amendment, liming agent and fertilizer has become increasingly popular. When applied to agricultural fields, MC can be introduced and can potentially accumulate in the soil and crops – creating a new pathway for human exposure (Ai et al., 2020).

Once in the soil, MCs adsorb onto soil surfaces due to chemical binding with the metal ions present (Bouaïcha and Corbel, 2016). Adsorption of MC is dependent on the clay content and soil type, as one of the important binding mechanisms is with the metal ions of soil particles (Chen et al., 2006). They have been reported to provoke structural and physiological changes in bacterial communities, largely due to the secondary antibacterial metabolites they produce (Bouaïcha and Corbel, 2016; Lahrouni et al., 2012). Additionally, they stimulate reactive oxygen species (ROS), ultimately affecting the proteins, lipids, and nucleotides of bacteria (Xiang et al., 2019). Soil microorganisms are sensitive to environmental stressors; therefore, their response is indicative to the overall soil health and quality.

Using 3-methoxy-2-methyl-4-phenylbutyric acid (MMPB), an oxidized product of microcystin, Foss and Aubel (2015) developed a method to determine the concentration of MC in treated and untreated water samples, but there are no methods that have accurately measured MC concentrations in WTR. It has been established by Ai et al. (2020) that MC is taken up in the

fruiting bodies of some agricultural crops. Therefore, it is essential to develop a method for WTR in order to discern those that are safe for agricultural application from those that are not due to MC contamination.

This paper developed a method for quantifying total MCs in WTR as well as investigated the effects MC and WTR have on soil microbial communities after incubating amended soil for 4 weeks.

2. Material and Methods

2.1 Soil Collection and Characterization

Two types of agricultural soils (0-15cm) were collected from Moser Farms in Northwestern Ohio (Clay Loam) and Nester Ag. in Toledo, Ohio (Sandy Loam). The clay loam is classified as a Mermill-Aurand complex (*Web Soil Survey, 2021)*, the sandy loam can be classified as a Rawson sandy loam. The sampled soil was stored in plastic bags and kept on ice overnight until they were wet sieved (<2mm). A subsample of the <2mm fraction was air dried for soil characterization. A subsample of the air dried <2mm fraction was pulverized when necessary for analysis. The clay loam and the sandy loam have both undergone a reduced conventional tillage and fertilizer management. In the fall, monoammonium phosphate and potash fertilizer are applied along with a 28% urea ammonium nitrate starter and sidedress. At the time of sampling, the clay loam was growing wheat and the sandy loam had been seeded with soybeans. The soil characteristics for both soil types are shown in Table 1. The clay loam can be characterized as 35.1% sand, 36.7% silt and 28.2% clay with a pH and EC of 6.75 and 0.241 dS m^{-1}, respectively. Additionally, the clay loam has a total CEC of 7.35 cmol kg^{-1} soil and 47.58 mg kg^{-1} of plant available P. Its total carbon and nitrogen content is 2.75% and 0.23%,

117 respectively. The sandy loam can be characterized as 66.7% sand, 20.6% silt and 12.7% clay
118 with a pH and EC of 6.01 and 0.178 dS m^{-1}, respectively. The sandy loam's total CEC is 6.04
119 cmol kg^{-1} soil, with 62.26 mg kg^{-1} of plant available P. The total carbon and nitrogen for the
120 sandy loam are 2.09% and 0.18%, respectively. Baseline microbial biomass (total PLFA),
121 microbial groups indicated by PLFA biomarkers, and arylsulfatase activities for both soils can be
122 found in the supplemental section (Table 6).
123
124 *2.2 Incubation Study Experimental Design*
125 Thirty-two glass beakers (300 mL) were prepared to conduct a non-destructive incubation
126 study. Beakers were filled with 100 g of sieved field soil (<2mm). The soils were preincubated
127 for one week at 23℃ before treatments were added. There were 4 soil replicates with the
128 following treatments: control, microcystin (MC), water treatment residual (WTR) and both WTR
129 and MC. The application rate of WTR, 10 tons per acre of soil, was based on personal
130 communication with the facilities manager from the water treatment plant it was collected from.
131 All soil treatments were incubated for 4 weeks.
132 Microcystin concentrations were derived using the WTR application rate as well as the largest
133 concentration from WTR 2 which was determined using the $NaHCO_3$/ACN MMPB extraction
134 method discussed later in this study. Given these rates, 0.0112 g of WTR and 0.0207 µg of MC
135 were applied per gram of soil for the samples that received treatments. The samples were kept at
136 23℃ at two-thirds field moisture for their allotted time periods. Subsamples for were kept at 4℃
137 and -20℃ for enzyme assays and PLFA analyses, respectively.
138
139

2.3 Water Treatment Residual Collection and Characterization

Eight different water treatment residuals were collected in 2018 from Water Treatment Plants across Ohio in Wilmington (WTR 1), Celina (WTR 2, collected July 2018), Sidney (WTR 3), Ottawa (WTR 4), Oregon (WTR 5), Celina (WTR 6, collected September 2018), Huron (WTR 7), and Toledo (WTR 8). Samples were collected into buckets out of the lagoon using a shovel. Following collection, the samples were air dried and then crushed and homogenized using a cement mixer. The homogenized WTR was then sieved through a <2mm sieve and a subsample was pulverized when necessary for analysis. For the following incubation study, WTR 3 will be used as one of the treatments because it had an undetectable concentration of MC. The characteristics of WTR 3 are displayed in Table 2. Water treatment residual 3 can be characterized as 6.0% sand, 87.0 silt, 7.0% clay with a pH and EC of 9.62 and 0.606 dS m^{-1}, respectively. Additionally, it contains 11.2% C and 0.012% N.

2.4 Microcystin Extraction Method and Quantification

A 1M $NaHCO_3$/DI Water/ACN solution was used for a total extraction of MC from WTR. For MC extraction, one gram of WTR was weighed into 15 mL centrifuge tubes. Four mL of extraction solution were added to the samples and were vortexed for about a minute per sample. Subsequently, the samples were added to a sonication bath for 20 minutes before they were centrifuged at 3500 rpm for 30 minutes. The supernatants were collected into their respective scintillation vials and the previous five steps were repeated as a rinse step. This resulted in a total of 8 mL of combined supernatant. The samples were dried down using N_2 gas and brought back to 8 mL volume using deionized water. Following, 1 mL of 500mM $KMnO_4$ and 1 mL of saturated $NaIO_4$ were added to each vial and were left in the dark to oxidize for 2

hours. Immediately after oxidation, the reaction was quenched by adding 40% $NaHSO_3$ (dropwise), swirling in between drops to fully mix the solutions. Oasis HLB 6cc/150mg extraction columns were prepared by conditioning with 3 mL of methanol followed by 3 mL deionized water using a vacuum pump. The samples were collected onto the columns using a mechanical vacuum extractor and were rinsed using 1 mL of 5% methanol. To release the MMPB, from the cartridge, 3 mL of 2% formic acid was applied and the eluent was collected in a vial. The samples were brought to dryness using N_2 gas and then reconstituted with 990 µL of methanol and 10 µL of the internal standard, 4-PB. The samples were run on a Thermo Quantiva Triple Quadrupole Mass Spectrometer using a ACQUITY UPLC HSS PFP Column (100Å, 1.8 µm, 2.1 mm X 100 mm) and two solvents, 500mL 5% methanol in 0.05% acetic acid along with 500mL 95% methanol in 0.05% acetic acid.

Using microcystin-LR, a 5-point standard curve was created using a range of concentrations (5, 10, 50, 100, and 200 ppb) to assess the method. Furthermore, samples that were spiked for quality control purposes, were spiked with MC-LR.

2.5 Soil and WTR Physical Methods

Soil texture was determined using a Hydrometer as previously described by Gee and Bauder (1986).

2.6 Soil and WTR Chemical Methods

Electrical conductivity (EC) was determined as previously described by Rhoades (1996). pH was determined as described by Thomas (1996). CEC was determined using a combination of procedures as previously described by Blakemore et al. (1981) and Ketterings et al. (2014).

Plant available P was determined by the Mehlich 3 analysis as previously described by Mehlich (1984). Total carbon and nitrogen were determined as previously described by Nelson and Sommers (1996).

2.7 Soil and WTR Biological Methods

Soil microbial PLFAs were determined as previously described by Frostegård et al. (1993) and Bardgett et al. (1996). Briefly, 2 g of soil was extracted using a chloroform, methanol and citrate buffer one-phase solution. Phospholipids, neutral lipids and glycolipids were collected in the organic phase and separated on a solid phase silicic acid extraction column (Supelco, LC-Si SPE tubes). The phospholipids (PLFAs) were collected and then methylated using alkaline methanolysis. PLFAs were analyzed by GCFID using an Agilent 6890N GC with a 25 m Ultra 2 column. Individual peaks were identified using a fatty acid standard and a MIDI library (MIDI Inc., Newark, DE). PLFA concentrations were quantified by adding an internal standard (methyl nonadecanoate; 19:0). Absolute concentrations of individual PLFAs were expressed as µmol PLFA g^{-1} dry soil. The summed masses of PLFAs reported as typical of saprophytic fungi, gram-negative bacteria, gram-positive bacteria, actinobacteria, and arbuscular mycorrhyzal fungi (AMF) were used as signatures for these microbial groups (Table 3).

Arylsulfatase activity was determined as previously described by Tabatabai (1994). Briefly, two 1 g soil samples were incubated at 37 ºC for 1 hr with *p*-nitrophenyl sulfate (Sigma N3877) substrate under pH buffered conditions. One 1-g control, a sample with soil and reagents, but without the addition of substrate was run for each sample. After incubation, the reaction was stopped in all assays using 4 ml THAM (tris-hydroxymethyl aminomethane) pH 12 and 1 ml of 0.5 M $CaCl_2$. The samples were then filtered through Whatman #2 filter paper. The

absorbance of the filtrate was measured using a spectrophotometer at 415 nm. The absorbance value of the control was subtracted from both replicates, and the two replicates averaged. Enzyme assays were rerun if variability between replicates exceeded 5%. A calibration curve was developed using standards containing 1, 100, 200, 300, 400, or 500 nmol *p*-nitrophenol in MUB diluted in a 1:1 mixture of MUB pH 6.0 and 0.1 M THAM pH 12. When absorbance values exceeded the highest p-nitrophenol standard value, the colorimetric solution was diluted until obtaining an absorbance within the standard curve.

2.8 Statistical Analyses

All physical, chemical and biological data was presented on a soil dry weight basis and was tested for normality (D'Agostino-Pearson test) prior to statistical analysis. PLFA and arylsulfatase data was compared between treatments using One-way ANOVA with Tukey's Honest Significant Difference Test ($p < 0.05$) for each soil type. PLFA and arylsulfatase data correlations were calculated using R Studio for Windows (version 1.4.1717).

3. Results

3.1 Microcystin (MC) Concentrations in Water Treatment Residual (WTR)

Total concentrations of MC found in each WTR are given in Table 4. Water treatment residual 2 and WTR 6 resulted in the largest concentration of total MCs, 1855.8 and 1050.1 ug kg^{-1}, respectively. Only one sample, WTR 3, had no detectable concentration of MC which was denoted as <20.0 ug kg^{-1}. The remaining samples, WTR 1, WTR 4, WTR 5, WTR 7, and WTR 8 had total MC concentrations of 100.0, 289.8, 50.4, 47.2, and 333.6 ug kg^{-1}, respectively. When

231 spiked with 500 ug kg^{-1} MC-LR, WTR 8 resulted in a total MC concentration of 837.9 ug kg^{-1},
232 for a 100.86% recovery.
233
234 *3.2 Soil Chemical Parameters (pH, EC, CEC, P, tC, tN) after 4 Weeks of Soil Incubation*
235 Both soils, the clay loam and the sandy loam showed an increase in pH after either
236 treatment with WTR was applied. After the addition of the WTR, MC and WTR+MC treatments,
237 the pH of the clay loam significantly increased ($p < 0.05$) from 6.53 to 7.73, 6.72 and 7.8,
238 respectively. Similarly, the pH of the sandy loam significantly increased ($p < 0.05$) from 5.88 to
239 7.72, 6.10 and 7.84 following the application of WTR, MC and WTR+MC, respectively (Tables
240 5 and 6).
241 After 4 weeks incubation, the electrical conductivity (EC) of both soils, the clay loam and
242 sandy loam significantly increased ($p < 0.05$) after application of the WTR and WTR+MC
243 treatments. In the clay loam, the addition of WTR and WTR+MC increased the EC from 0.241
244 dS m^{-1} to 0.508 and 0.441 dS m^{-1}, respectively. As for the sandy loam, the EC increased from
245 0.315 dS m^{-1} to 0.564 and 0.448 dS m^{-1} after WTR and WTR+MC were applied, respectively.
246 Importantly, with the application of the MC treatment, the EC of the clay loam and sandy loam
247 significantly decreased ($p < 0.05$) to 0.188 and 0.182 dS m^{-1}, respectively (Tables 5 and 6).
248 There was no significant change in cation exchange capacity (CEC) for the clay loam
249 after the application of any of the three treatments. In the sandy loam, there was a significant
250 increase ($p < 0.05$) in CEC following the application of the WTR and WTR+MC treatments. The
251 MC treatment exhibited a significant decrease ($p < 0.05$) in CEC after the 4-week incubation
252 (Tables 5 and 6).

The WTR and WTR+MC treatments exhibited the greatest decrease in plant available P in the clay loam and sandy loam. In the clay loam, available P concentrations significantly decreased ($p < 0.05$) from 51.26 mg kg^{-1} to 46.92 and 45.35 mg kg^{-1} after application of the WTR and WTR+MC treatments, respectively. The MC treated soil exhibited a slight decrease in available P concentration but was not significant. However, in the sandy loam, the concentration of available P in the WTR, MC and WTR+MC treated soils significantly decreased ($p < 0.05$) from 65.17 mg kg^{-1} to 58.07, 63.34 and 57.64 mg kg^{-1}, respectively (Tables 5 and 6).

Following the application of the WTR, MC and WTR+MC treatments in the clay loam, there was no significant change in the total C. Additionally, there was no significant change in total C in the sandy loam for the MC or WTR+MC treatments. However, there was a significant increase ($p < 0.05$) in total C in the WTR treatment in the sandy loam. There were no significant changes in total N in either soil type for any of the treatments (Tables 5 and 6).

3.3 Microbial Parameters after 4 Weeks of Soil Incubation

3.3.1 Soil Arylsulfatase activity

None of the treatments in the clay loam exhibited any significant change in arylsulfatase activities after 4 weeks. In the sandy loam, the MC treatment exhibited a significant increase ($p < 0.05$) in arylsulfatase activity compared to the control group at 4 weeks. In addition, the WTR+MC treatment significantly increased arylsulfatase activities compared to the control group ($p < 0.05$). The WTR treatment had no significant effect on arylsulfatase activities in the sandy loam when compared to the control (Figure 1).

3.3.2 Soil Phospholipid Fatty Acids

Total PLFA concentrations and PLFA biomarker concentrations for microbial groups such as gram-negative bacteria, gram-positive bacteria, Actinobacteria, protozoa and fungi (saprophytic and arbuscular mycorrhiza) are shown on Table 7. Total soil microbial phospholipid fatty acids (total PLFA) significantly increased to the largest extend after MC treatment ($p < 0.05$) compared to the control, followed by the WTR+MC treatment. The WTR treatment alone did not reveal any significant increase of total PLFA compared to the control. Similarly, the WTR+MC and MC treatments exhibited a significant increase ($p < 0.05$) compared to the control in the sandy loam, but they were not as sizeable as the clay loam. There was no significant increase in total PLFA concentration following the application of the WTR treatment. Overall, the clay loam had larger concentration of total PLFA than the sandy loam.

PLFA biomarkers for gram-negative bacteria in the clay loam increased for all treatments compared to the control, but only the MC and WTR+MC treatments were statistically significant ($p < 0.05$). As for the sandy loam, there was a significant increase in the MC, WTR and WTR+MC treatments ($p < 0.05$) compared to the control. The overall gram-negative bacteria concentrations are notably higher in the clay loam compared to the sandy loam.

Gram-positive bacterial biomarkers significantly increased in the MC treatment ($p < 0.05$) compared to the control group in the clay loam. As for the sandy loam, there were no significant differences in gram-positive bacteria concentration for any of the treatments. Importantly, there is a higher concentration of gram-positive bacteria in the clay loam soil versus the sandy loam.

In the clay loam, only the MC treatment significantly increased Actinobacteria ($p < 0.05$) from the control group. The WTR treatment exhibited an increase in Actinobacteria

concentration after 4 weeks, but it was not significant. There were no treatments in the sandy loam soil that were significantly different from the control group.

At 4 weeks, there was a significant increase ($p < 0.05$) in saprophytic fungal concentration following the MC treatment in the clay loam. There were slight increases seen in the WTR and WTR+MC treatments, but they were insignificant. There were no significant differences in saprophytic fungal concentration in any of the treatments in the sandy loam compared to the control group. In general, the sandy loam did not have as large of saprophytic fungi concentrations as the clay loam.

The arbuscular mycorrhiza fungi (AMF) biomarker at the 4-week time period of the MC treatment in the clay loam was significantly different from the control group ($p < 0.05$). While the WTR and WTR+MC treatments slightly increased compared to the control group, they were not significantly different. As for the sandy soil at 4 weeks, the MC, WTR and WTR+MC treatment significantly increased AMF ($p < 0.05$) compared to the control group. There was a notable difference in AMF concentration between the two soil types, with the clay loam having much larger concentrations at both time periods.

Protozoa biomarkers were not significantly different in treated soils compared to the control in the clay loam. The MC and WTR+MC treatments both significantly increased ($p < 0.05$) the concentration of Protozoa compared to the control group in the sandy loam soil.

After 4 weeks incubation, all treatments in the clay loam significantly increased the gram-negative / gram-positive ratio ($p < 0.05$) compared to the control. The largest increase was detected in the WTR+MC treatment, followed by the MC and WTR treatment. The sandy loam followed a similar trend with a significant increase ($p < 0.05$) exhibited in the MC, WTR and WTR+MC treatments compared to the control group. In the clay loam and sandy loam, all

treatments exhibited a significant decrease ($p < 0.05$) in the 17:0cycc+c19:0cyc to 16:1w7c+18:1w7c microbial stress ratio. The most notable decreases were seen in the WTR+MC and MC treatments. No significant changes of the fungal/bacterial ratio were detected for any treatment in both soils, the clay loam and sandy loam.

4. Discussion

4.1 Microcystin Concentrations detected in WTR from MMPB Extraction Method.

This study was prompted by the research conducted by Foss and Aubel (2015) in which total MC detected in water samples by the MMPB UPLC-MS/MS method was compared to the enzyme-linked immunoassay (ELISA) method. Foss and Aubel (2015) found that the ELISA method overestimated the concentration of MC in the water samples compared to the MMPB method in many samples. During this study, it was determined that the MMPB method is an appropriate method to determine MC concentrations in WTR since there was a 100% spike recovery. While ELISA was used by Ai et al. (2020) to measure concentrations of MC in WTR, this data in questionable since it was not verified by quality control measures, and therefore, the determined concentrations may have resulted in an overestimation.

4.2 Chemical Properties Following a 4-Week Incubation.

While WTR is typically used as a liming agent, the pH levels of these residuals do vary by water treatment facility. In this study, the WTR used was very alkaline, so application to both soils increased their pH. Some WTR's, such as the ones used in Lombi et al. (2010), are neutral and therefore, do not affect the soil pH after application. The significant increase in pH in the clay loam and sandy loam following the MC treatment should be further investigated.

The EC of the WTR was 60.2% and 70.6% greater than the EC of the clay loam and sandy loam, respectively. Additions of WTR in either soil after 4 weeks increased the overall salinity of that soil. A similar increase in EC was seen after application of WTR from a study conducted by Novak and Watts (2005). Furthermore, the WTR+MC treatment increased EC, but not as much as the WTR treatment alone, likely due to MC's ability to decrease the EC. Therefore, further investigation should be conducted in order to understand the decrease in EC following the application of the MC treatment.

The notable reduction in available P concentrations in the WTR and WTR+MC treated soils can be attributed to the ability of WTR to tightly adsorb P (Dayton et al., 2003). Many microbial groups increased with the addition of MC to the soil. The growth of soil microbial biomass indicated by an increase in total PLFA after MC treatment could have caused a slight decrease in available P concentration in the MC treated soil, as microorganisms will consume P. Furthermore, this microbial P consumption could account for the additional available P loss in the WTR+MC combination treatment compared to the WTR treatment alone.

There was no significant difference in CEC for any of the treatments applied to the clay loam. Depending on the clay minerals, soils high in clay content will have a larger CEC fraction than those with a large percent sand or silt content (Parfitt et al., 1995). Additions of WTR increased the CEC in the sandy soil. This can be attributed to sand having a negligible CEC and relatively no buffer capacity. Adding WTR, a substrate likely high in cations from the water treatment process, will increase the CEC of a sandy soil. Similarly, an increase in CEC following the addition of biosolids to soil was seen in a study conducted by Neilsen et al. (2003). The significant decrease in CEC following the application of MC in the sandy loam is intriguing and should be investigated further.

4.3Microbial Parameters after 4 Weeks of Soil Incubation

4.3.1 Soil Arylsulfatase activity

The enzyme arylsulfatase is produced by microorganisms and plant roots, and its activity is used as a measure for potential biochemical mineralization of organic ester sulfates in the soil (Farrell et al., 1994). Mineralization of organic ester sulfates is essential for microorganisms in order to release sulfur, and therefore, access carbon for energy (Gahan and Schmalenberger, 2014). The clay loam did not have any notable shift in arylsulfatase activities for any of the treatments. Due to the sorption capacity of clayey soils, arylsulfatase was likely stored in the minerals as a clay-enzyme complex therefore, having no effect on the enzyme reactivity (Tietjen and Wetzel, 2003). Another explanation could be that arylsulfatase activities are lagging and might show changes after a longer time period. The sandy loam showed a large increase in arylsulfatase activity in response to the MC treatment. Sandy soils have a smaller background of enzyme storage due to its poor adsorption capacity. Therefore, with the increase in saprophytic and arbuscular mycorrhizal fungal community after the addition of MC, there was likely a larger production of arylsulfatase and this increased arylsulfatase activity as means to access more carbon (Gahan and Schmalenberger, 2014).

4.3.2 Phospholipid Fatty Acids

The WTR+MC and MC treatments both significantly increased the total PLFA concentrations in the clay loam and the sandy loam. The MC treatment exhibited the largest increase in total PLFA concentration in the clay loam. This is in contrast to a study conducted by Haney et al. (2000), where the application of glyphosate to soil did not significantly affect soil

391 microbial biomass C and N. Additionally, while the total PLFA concentrations increased
392 following the addition of WTR, the increase was not as notable as the increase after application
393 of sewage sludge in the study conducted by Börjesson et al. (2014). The WTR+MC combination
394 treatment exhibited a larger increase in total PLFA than the WTR treatment, but less than the
395 MC treatment alone. This suggests that the WTR could be inhibiting microbial growth due to the
396 collection of impurities during its production as well as its ability to reduce available P
397 (Gallimore et al., 1999; Ippolito et al., 2011).

398 For both soil types, there was a significant increase in gram-negative bacteria
399 concentrations for all treatments compared to the untreated controls. The most notable increases
400 were in the treatments that contained MC. Gram-negative bacteria are closely associated with
401 labile carbons, such as those that are derived by plants (Fanin et al., 2019). Microcystin is a
402 relatively simple structure, with only one ring structure on the Adda moiety tail (Figure 2). The
403 addition of MC seems to be creating a positive environment for gram-negative bacteria, by
404 providing them with an available carbon source. If true, this information would contradict the
405 statement of Xiang et al. (2019) in which MC is a recalcitrant substrate and therefore, difficult
406 for microorganisms to break down. While the WTR treatment stimulated an increase in gram-
407 negative bacteria concentration, it was not as sizeable compared to the other two treatments.

408 There was not much of an increase in gram-positive bacteria concentration in the clay
409 loam soil. While there was a significant, but small increase in concentration from the MC
410 treatment and WTR+MC treatment. Gram-positive bacteria are associated with recalcitrant
411 carbon compounds that are difficult for organisms to break down (Fanin et al., 2019). The lack of
412 response in gram-positive bacteria concentrations from the treatments suggests that neither MC
413 nor WTR are recalcitrant sources of carbon.

The increase in Actinobacteria concentration in the clay loam soil at 4 weeks is likely due to Actinobacteria's ability to adapt to changes in environmental conditions due to their characteristic growth pattern resembling fungal hyphae enabling them to spread in soil effectively. The treatments in both the clay loam and sandy loam that contained WTR exhibited insignificant increases in Actinobacteria concentration. While this supports the results of Frostegård et al. (1993) who found an increase in Actinobacteria following the addition of liming material, the increases were insignificant. The Actinobacteria concentrations in both the clay loam and sandy loam, exhibited a similar response to the treatments as the fungal groups, suggesting their similarities.

Soil fungal communities are essential for soil aggregation and nutrient cycling through decomposition of organic matter (Kaur et al., 2005; Veum et al., 2019). There was small increase in saprophytic fungi following the application of MC in the clay loam. While there are no studies that have looked at the effects of MC on fungal communities, this can be compared to glyphosate, a common herbicide applied in agricultural systems. Lane et al. (2012) found no effect on the saprophytic fungal community following the application of glyphosate. Similar to the study conducted by Börjesson et al. (2014) using sewage sludge as a soil amendment, there was no effect on the fungal community following the application of WTR.

Although there was a significant increase in AMF concentration in the MC treated clay and sandy loam soil, as well as the WTR and WTR+MC treated sandy loam soil, this is likely due to the contribution of bacteria with a similar biomarker as AMF, such as *Cytophaga* and *Flexibacter* (Yu et al., 2021). Arbuscular mycorrhizal fungi are obligate symbionts, therefore, in theory, they need a host plant in order to expand their network as they are not free living (Berruti et al., 2016). At the time of sampling, wheat was growing in the clay loam and soybeans had

been planted in the sandy loam, but during incubation, there were no plants growing. Any concentration detected should be attributed to residual AMF fatty acid biomarkers left it the soil.

The predation of bacteria by Protozoa is essential in stimulating bacterial growth as well as mineralizing nitrogen (Clarholm, 1981). There was not much change in Protozoa concentration in the clay loam. There was a notable increase in the sandy loam following the WTR+MC and MC treatment. Given the lack of bacterial response in the sandy loam after application of treatments, the increase in Protozoa concentration is unexpected as they feed on bacterial groups. However, a possible explanation would be that the reaction of Protozoa is lagging, a later sampling time might have shown an increase of Protozoa.

Changes in the gram-negative to gram-positive bacterial ratios have been used as an indicator of environmental stress as well as shifts in soil organic C availability (Willers et al., 2015; Fanin et al., 2019). For both the clay loam and the sandy loam, all treatments exhibited an increase in the gram-negative to gram-positive bacteria ratio, but only the WTR+MC and MC treatments were significant. An increase in gram-negative bacteria is often associated with stressful conditions such as osmotic stress or heavy metal contamination (Willers et al., 2015; Veum et al., 2019). Furthermore, an increase in this ratio can suggest the addition of labile carbon compounds as gram-negative bacteria are known to obtain their energy from easily decomposable carbon (Fanin et al., 2019).

Gram-negative bacteria produce elevated levels of monounsaturated fatty acids (16:1w7c and 18:1w7c) during the growth phase when their metabolism is active. Contrarily, when their metabolism slows down, gram-negative bacteria transform unsaturated fatty acids to cyclopropane fatty acids (17:0cyc and c19:0cyc) (Veum et al., 2019). A larger ratio of cyclopropane fatty acids to monounsaturated fatty acids can be indicative of various

environmental stressors. In both the clay loam and the sandy loam, all treatments exhibited a decrease in the stress ratio, but it was most notable in the WTR+MC and MC treatments. This is likely due to the available carbon source the MC was providing for the microbial community, as increasing the microbe's available energy source would decrease the stress they may be experiencing. Another explanation could be that an increased soil pH after WTR treatment was beneficial for soil bacteria.

The fungal to bacterial biomarker ratio is used as an indicator for environmental changes such as shifts in the carbon to nitrogen dynamic, decomposition of organic matter and carbon sequestration (Willers et al., 2015; Zhang et al., 2016; Veum et al., 2019). Fungal communities prefer a higher C:N ratio, therefore, a decrease in fungal to bacterial ratios is associated with a decrease in C:N ratio (Zhang et al., 2016). Only the WTR treatment in the clay loam exhibited a slight increase in the fungal to bacterial biomarker ratio. Following the addition of all treatments, there was not a significant change in the C:N ratios. Furthermore, there was a consistent increase in bacteria and fungi for each treatment and soil type, which would result in similar fungal to bacterial ratios as the control.

5. Conclusions

This study successfully developed and verified the MMPB method as an accurate measurement of MC concentration in WTR. For future research, this technique should be used on the 4-week incubation samples in order to determine the degradation of MC by microorganisms. Obtaining this information will provide further explanation of the discoveries gathered in this investigation.

Application of MC and WTR+MC to both soils significantly increased total PLFA, gram-negative bacteria, and total fungal concentrations. There was a clear pattern for other parameters as well underlining a stimulative effect of WTR and MC on the soil microbial community. For future research backup soil samples at 4 weeks were obtained and have been stored in -80℃ for possible DNA extraction to analyze specific changes in microbial species following the application of WTR and MC treatments. Furthermore, it would be beneficial to analyze additional enzyme assays, in order to comprehend how MC may be affecting microbial processes necessary for adequate soil and plant health.

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Tables and Figures

Table 1. *Soil characteristics of the clay loam and sandy loam. EC, Electrical Conductivity. CEC, Cation Exchange Capacity. P, Phosphorus. C, Carbon. N, Nitrogen.*

	pH	EC	CEC	Available P	C	N
		[dS m^{-1}]	[cmol kg^{-1} soil]	[mg kg^{-1}]	[%]	[%]
Clay Loam	6.75	0.241	7.35	47.58	2.75	0.23
Sandy Loam	6.01	0.178	6.04	62.26	2.09	0.18

Table 2*. Characteristics of the Water Treatment Residual. EC, Electrical Conductivity. C, Carbon. N, Nitrogen.*

	pH	EC	Texture	C	N
		[dS m^{-1}]		[%]	[%]
WTR 3	9.62	0.606	Silt loam	11.2	0.012

706 Table 3. Taxonomic microbial groups with the PLFA group and specific PLFA markers used to
707 designate such groups.

Taxonomic group	PLFA group	Specific PLFA markers	Reference
Bacteria	multiple groups	sum of i15:0, a15:0, i16:0, i17:0, a17:0, cy17:0, cy19:0, 16:1ω7, 18:1ω7, and 17:1ω9	Frostegard and Bååth, 1996
GM+	branched PLFAs	sum of i15:0, a15:0, i16:0, i17:0, and a17:0	O'Leary, 1988
GM -	cyclopropyl and mono PLFAs	sum of cy17:0, 16:1ω7, 18:1ω7, and 17:1ω9	Wilkinson, 1988
Actino-bacteria	10Me-PLFAs	sum of 10Me16:0, 10Me17:0, and 10Me18:0	Kroppenstedt, 1985
Fungi	polyunsaturated PLFAs	18:2ω6,9	Federle et al., 1986; Frostegard and Bååth, 1996
F:B ratio	multiple groups	Fungi/Bacteria	Federle et al., 1986; Frostegard and Bååth, 1996
Protozoa	polyunsaturated PLFAs	sum of 20:2ω6 and 20:4ω6	Ringelberg et al., 1997
Arbuscular mycorrhizal fungi (AMF)	16:1ω5c	Also present in some bacteria as *Cytophaga* / *Flexibacter*; also a major source of storage (neutral lipids) in AMF spores and vesicles.	Nichols et al., 1986; Olsson et al., 1995; van Aarle et al., 2003; Walker, 1969..
Microbial Stress Ratio	17:0cyc+19:0cyc / 16:1w7c+18:1w7c		Petersen and Klug, 1994 Veum et al., 2019
GM- / GM+	cyclopropyl and mono PLFAs / branched PLFAs		Petersen and Klug, 1994 Willers et al., 2015 Fanin et al., 2019

GM+ = Gram-positive bacteria
GM - = Gram-negative bacteria

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Table 4. *Total concentration of MMPB (ug kg^{-1}) found in each WTR sample and the corresponding spike recovery using a 1M $NaHCO_3$ and 1M ACN extraction solution.*

Water Treatment Residual	**MMPB**
	[*ug kg^{-1}*]
WTR 1	100.0
WTR 2	1855.8
WTR 3	<20.0
WTR 4	289.8
WTR 5	50.4
WTR 6	1050.1
WTR 7	47.2
WTR 8	333.6
WTR 8, Dupe Spike	837.9
WTR 8, Dupe Spike Recovery	504.3
Spike Amount	500.0

Table 5. *Standard soil characteristics for the clay loam after undergoing 4 weeks of respective treatment.*

	Control	WTR	MC	WTR + MC
pH	6.53 d	7.73 b	6.72 c	7.80 a
EC (dS m^{-1})	0.241 c	0.508 a	0.188 d	0.441 b
CEC (cmol kg^{-1} soil)	7.50 a	7.59 a	7.49 a	7.52 a
Available P (mg kg^{-1})	51.26 a	46.92 b	49.70 a	45.35 b
% C	2.72 a	2.88 a	2.76 a	2.79 a
% N	0.23 a	0.24 a	0.24 a	0.23 a

Significant differences between treatments are indicated by different letters (HSD groups, $p < 0.05$). WTR, Water Treatment Residual. MC, Microcystin. WTR+MC, Water Treatment Residual + Microcystin.

Table 6. *Standard soil characteristics for the sandy loam after undergoing 4 weeks of respective treatment.*

	Control	WTR	MC	WTR + MC
pH	5.88 d	7.72 b	6.10 c	7.84 a
EC (dS m^{-1})	0.315 c	0.564 a	0.182 d	0.448 b
CEC (cmol kg^{-1} soil)	6.23 b	6.57 a	6.00 c	6.42 a
Available P (mg kg^{-1})	65.17 a	58.07 c	63.34 b	57.64 c
% C	1.68 b	1.88 a	1.70 ab	1.78 ab
% N	0.16 a	0.17 a	0.16 a	0.16 a

Significant differences between treatments are indicated by different letters (HSD groups, $p < 0.05$). WTR, Water Treatment Residual. MC, Microcystin. WTR+MC, Water Treatment Residual + Microcystin.

Table 7. *PLFA biomarker concentrations (nmol g^{-1}) in two different soil types after undergoing different treatments for 4 weeks.*

Treatment	Gram +	Gram -	Actino	Protozoa	Sapr. Fungi	AMF	Total Fungi	Total PLFA	Gram-/Gram+	F:B	Microbial Stress Ratio
						Clay Loam					
Control	**14.73** b	**18.05** b	**7.84** b	**0.41** a	**6.66** b	**3.04** b	**9.70** b	**51.88** c	**1.23** c	**0.23** a	**0.70** a
WTR	**14.49** b	**19.17** b	**7.89** ab	**0.38** a	**7.97** ab	**3.03** ab	**11.00** b	**54.02** bc	**1.32** b	**0.26** a	**0.67** b
MC	**17.55** a	**25.90** a	**9.48** a	**0.39** a	**9.42** a	**3.65** a	**13.07** a	**67.77** a	**1.48** a	**0.24** a	**0.63** c
WTR+MC	**15.85** b	**23.99** a	**8.60** ab	**0.41** a	**8.06** ab	**3.37** ab	**11.43** ab	**61.52** ab	**1.51** a	**0.23** a	**0.61** c
						Sandy Loam					
Control	**12.20** a	**14.34** c	**7.02** a	**0.40** b	**6.20** a	**2.09** b	**8.29** b	**43.14** b	**1.17** d	**0.24** a	**0.66** a
WTR	**13.16** a	**16.88** b	**7.64** a	**0.42** b	**6.57** a	**2.49** a	**9.06** ab	**48.08** ab	**1.29** c	**0.24** a	**0.59** b
MC	**13.77** a	**18.98** ab	**7.58** a	**0.50** a	**7.10** a	**2.66** a	**9.76** a	**51.58** a	**1.38** b	**0.24** a	**0.56** c
WTR+MC	**13.62** a	**20.06** a	**7.62** a	**0.51** a	**7.13** a	**2.74** a	**9.86** a	**52.37** a	**1.47** a	**0.23** a	**0.51** d

Each bolded value is the mean of four replicates. Significant differences between treatments are indicated by HSD groups ($p < 0.05$). Gram +, Gram-positive Bacteria. Gram -, Gram-negative Bacteria. Actino, Actinobacteria. Sapr. Fungi, Saprophytic Fungi. AMF, Arbuscular Mycorrhizal Fungi. F:B, Fungal to Bacteria Ratio. Microbial Stress Ratio, 17:0cycc and c19:0cyc to 16:1w7c and 18:1w7c. WTR, Water Treatment Residual. MC, Microcystin. WTR+MC, Water Treatment Residual + Microcystin.

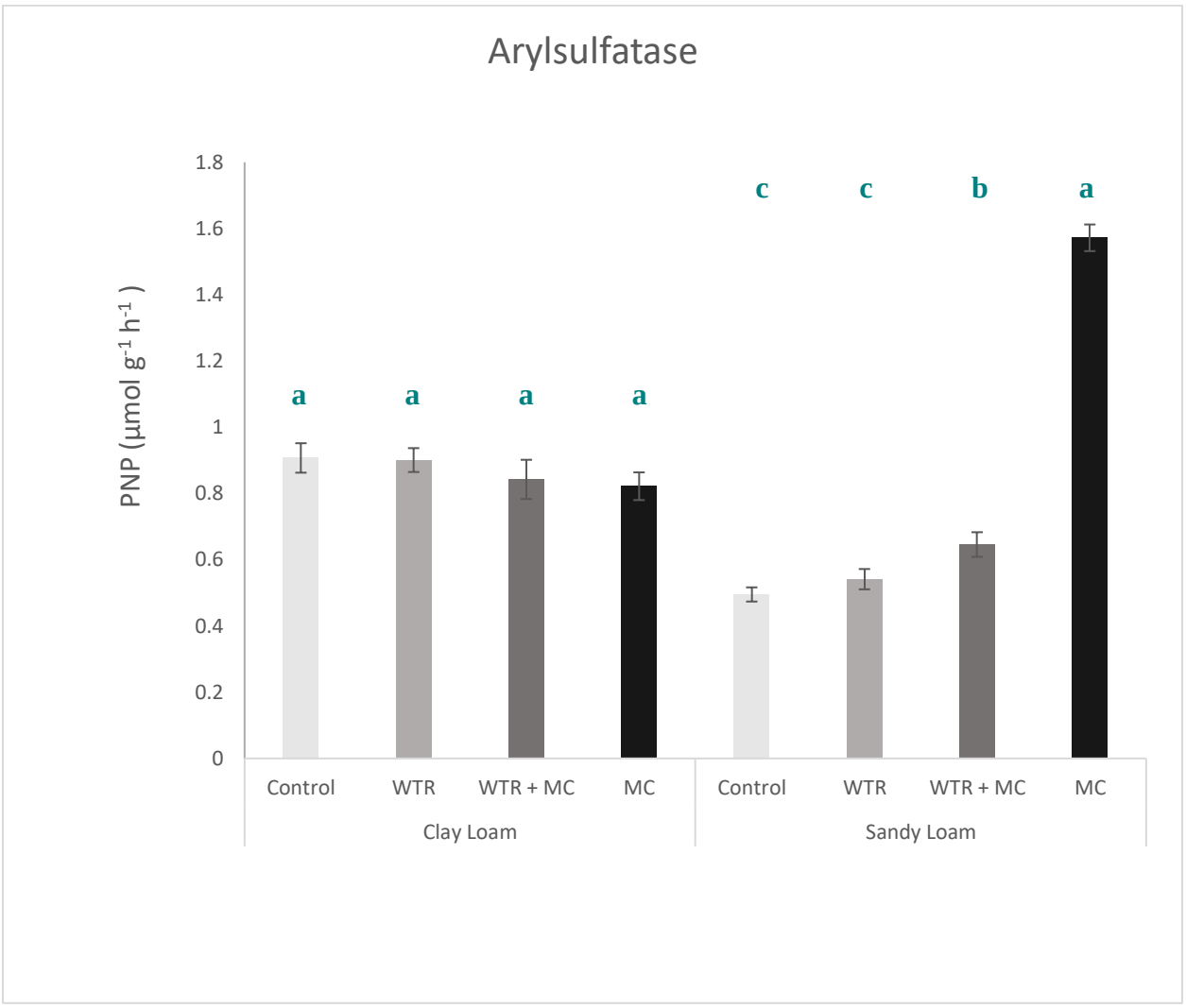

Figure 1. *Arylsulfatase activities in two different soil types after undergoing different treatments. Significant differences between treatments within one soil type are indicated by different letters (HSD groups, $p < 0.05$). WTR, Water Treatment Residual. MC, Microcystin. WTR+MC, Water Treatment Residual + Microcystin.*

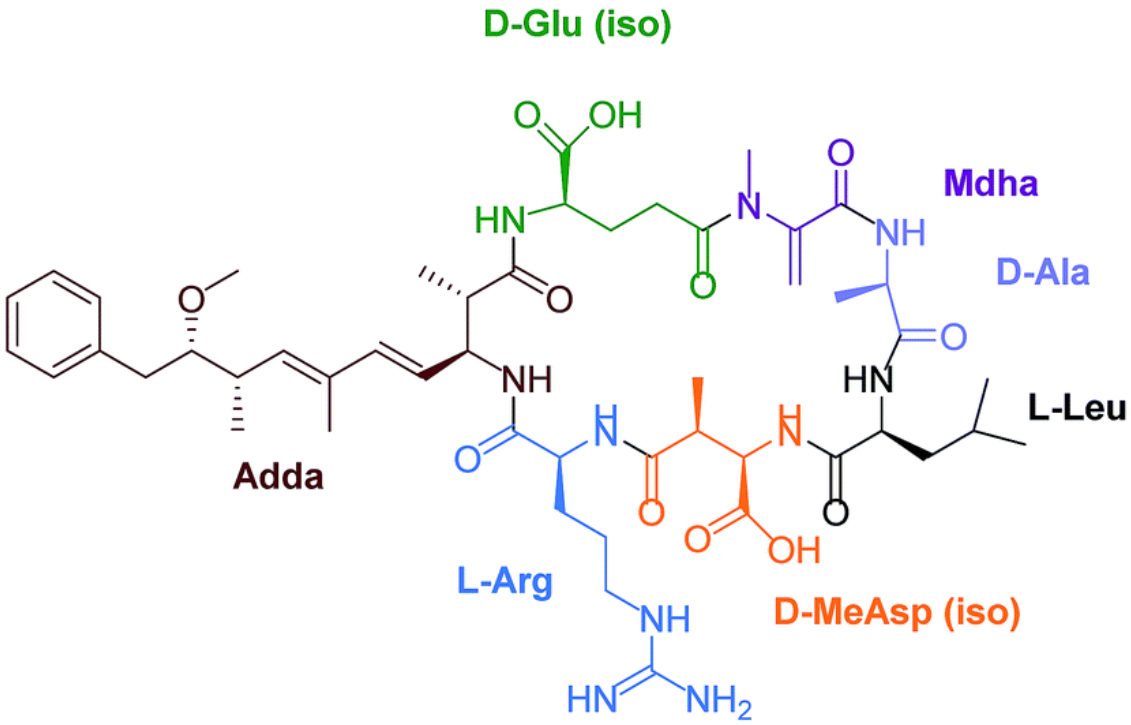

Figure 2. *Microcystin-LR chemical structure (borrowed from Neumann et al., 2016).*

Supplemental Information

Table 8. *Baseline microbial PLFA bioindicators and total PLFA (nmol g^{-1} soil) and Arylsulfatase activities (PNP µmol g^{-1} hr^{-1}) for clay loam and sandy loam. AMF, Arbuscular Mycorrhizal Fungi. PLFA, Phospholipid Fatty Acids.*

	Gram +	Gram -	Actinobacteria	Total Saprophytic Fungi	AMF	Protozoa	Total PLFA	Arylsulfatase
Clay Loam	13.60	19.54	7.86	6.70	2.77	0.31	51.80	1.08
Sandy Loam	9.89	12.96	6.27	5.16	1.84	0.24	37.16	0.64